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Current Opinion in Plant Biology

Beyond the photocycle — how cryptochromes regulate photoresponses in plants?

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Cryptochromes (CRYs) are blue light receptors that mediate light regulation of plant growth and development. Land plants possess various numbers of cryptochromes, CRY1 and CRY2, which serve overlapping and partially redundant functions in different plant species. Cryptochromes exist as physiologically inactive monomers in darkness; photoexcited cryptochromes undergo homodimerization to increase their affinity to the CRY-signaling proteins, such as CIBs (CRY2-interacting bHLH), PIFs (Phytochrome-Interacting Factors), AUX/IAA (Auxin/INDOLE-3-ACETIC ACID), and the COP1-SPAs (Constitutive Photomorphogenesis 1-Suppressors of Phytochrome A) complexes. These light-dependent protein–protein interactions alter the activity of the CRY-signaling proteins to change gene expression and developmental programs in response to light. In the meantime, photoexcitation also changes the affinity of cryptochromes to the CRY-regulatory proteins, such as BICs (Blue-light Inhibitors of CRYs) and PPKs (Photoregulatory Protein Kinases), to modulate the activity, modification, or abundance of cryptochromes and photosensitivity of plants in response to the changing light environment.

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Introduction

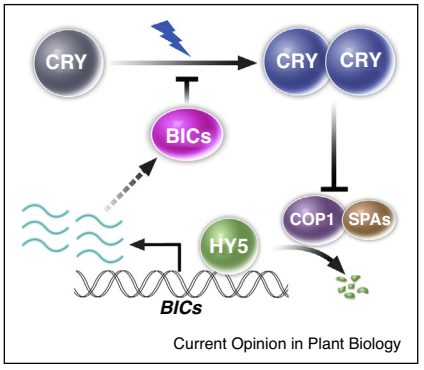
Cryptochromes mediate various blue light responses of plants [1,2], including changes to the transcriptome [3], inhibition of hypocotyl elongation [4], stimulation of cotyledon expansion [5], promotion of floral initiation [6], entrainment of the circadian clock [7], stimulation of stomata opening [8], fostering pathogen resistance [9], suppressing leaf senescence [10], inhibiting germination of dormant grain [11], regulating stomatal development [12], shade avoidance [13•], light-dependent stress responses [8,14,15,16•], and likely additional photoresponses yet to be discovered. Among various cryptochrome-mediated photoresponses in plants, the blue-light inhibition of hypocotyl elongation and photoperiodic promotion of floral initiation in Arabidopsis are the most extensively investigated [17]. Like other photoreceptors, photoexcited cryptochromes are expected to undergo photocycles, namely the reversible changes of the energy, orbital, or electronic state of chemical bonds of the FAD (Flavin Adenine Dinucleotide) chromophore. This signal perception process, regardless of its nature, is accompanied or followed by changes of the conformation of cryptochromes and their affinities to the CRY-binding proteins to initiate the signal transduction processes. The flavin photoreduction of cryptochromes, which was initially reported in Arabidopsis CRY1 [18], has been proposed to initiate a redox photocycle of cryptochromes via electron transfer through three conserved tryptophan residues referred to as the trp-triad [19,20]. However, the functional relevance of the trp-triad-dependent photoreduction of photolyase/cryptochrome protein family has been brought into question by the results of genetic studies of the trp-triad mutants of *Escherichia coli* photolyase [21•,22], insect cryptochromes [23•,24•,25], and Arabidopsis cryptochromes [26,27•]. In contrast to the lack of substantial advancement in our knowledge regarding the cryptochrome photocycle since our last review of this subject [28], significant progresses have been made in recent years to elucidate the signal transduction mechanisms of plant cryptochromes.

CRY photodimerization

The earliest light-induced and functionally relevant change of cryptochrome molecules is the light-dependent homodimerization and oligomerization. The recombinant and the endogenous Arabidopsis CRY2 proteins form 'nuclear speckles', also referred to as 'nuclear bodies' and 'photobodies', in the nuclei of plant cells exposed to blue light [29••,30]. The Arabidopsis CRY2 expressed in human HEK293 cells also forms morphologically similar photobodies in response to blue light [31•], suggesting that the CRY2 photobodies are composed of oligomerized CRY2 proteins. A series of elegant studies established that homodimerization or oligomerization is required for

the function of Arabidopsis CRY1 and CRY2 [32••,33••]. In the first study, it was investigated why the fusion proteins GUS-CCT1 and GUS-CCT2 exhibit constitutive photomorphogenic activity [32••]. It had been previously shown that transgenic expression of the GUS-CCT1 or GUS-CCT2 fusion proteins, which are β-glucuronidase (GUS) fused to the C-terminal CCT (also referred to as CCE for CRY C-terminal Extension) domains of CRY1 or CRY2 caused constitutive photomorphogenic phenotype similar to that of the *constitutive photomorphogenesis 1* (*cop1*) mutant [34•,35]. The ability of those fusion proteins to confer the constitutive photomorphogenic phenotype was later found to depend on oligomerization activity of the GUS moiety of GUS-CCT1 and GUS-CCT2 [32••]. It was further demonstrated that CRY1 and CRY2 form homodimers via their N-terminal CNT (also referred to as PHR for Photolyase Homologous Region) domains, that the CNT1 fragment can interact with the endogenous CRY1 to cause dominant-negative inhibition of the activity of CRY1 in transgenic plants, and that the CNT1 fragments of CRY1 mutated in A462V, G347R, or S66N, lost their activities to interact with CRY1 or to inhibit CRY1 activity *in vivo* [32••]. In the second study, it was shown that the chemically induced dimerization of the C-terminal domain fragments of CRY2 could elicit changes of expression of the CRY-target genes in the absence of light [33••]. These results demonstrate that homodimerization or oligomerization is necessary for the function of plant cryptochromes. Although no obvious blue light dependence was detected for the CRY1 or CRY2 dimerization in those earlier experiments [32••], the blue light-dependent homodimerization, also referred to as photodimerization, of Arabidopsis CRY1 and CRY2 has been recently detected in human HEK293 cells and in transgenic plants expressing near stoichiometric amounts of two recombinant CRY proteins fused to different epitope tags [36••,37] (Q. Wang, unpublished results). Cryptochrome photodimerization is apparently a regulated process in plant cells. Two closely related CRY inhibitory proteins, referred to as BIC1 and BIC2 (Blue-light Inhibitor of Cryptochromes 1 and 2), were identified in a genetic screen to search for negative regulators of cryptochromes [36••]. BICs inhibit blue light-dependent CRY2 homodimerization, oligomerization, and photobody formation. The loss-of-function *bic1bic2* double mutants are hypersensitive to blue light, whereas overexpression of BIC1 or BIC2 in transgenic plants suppressed all known photobiochemical and photophysiological activities of CRY1 and CRY2, including blue light inhibition of hypocotyl elongation, photoperiodic promotion of flowering, blue light-responsive gene expression, blue light-dependent interaction of cryptochromes with CRY-signaling proteins, blue light-dependent phosphorylation of CRY1 and CRY2, and blue light-dependent polyubiquitylation and degradation of CRY2 [36••]. A follow-up study showed that light induces transcription of the *BIC* genes in the cryptochrome-, phytochrome-, COP1- and HY5-dependent manner, suggesting not only a CRY-BIC negative feedback circuit but also a mechanism that may coordinate the co-action of phytochromes and cryptochromes [38]. Based on these results, a model of photoactivation and inactivation of plant cryptochromes was proposed (Figure 1). According to this model, cryptochromes exist as inactive monomers in the absence of light. In response to blue light, the photoexcited cryptochromes undergo conformational change to form homodimers and oligomers, which interact with CRY-signaling proteins to change gene expression and photomorphogenesis. As a negative feedback mechanism, cryptochromes positively regulate transcription of the *BIC* genes, which in turn bind to the photoexcited cryptochromes to inhibit CRY homodimerization and CRY activities (Figure 1).

Figure 1



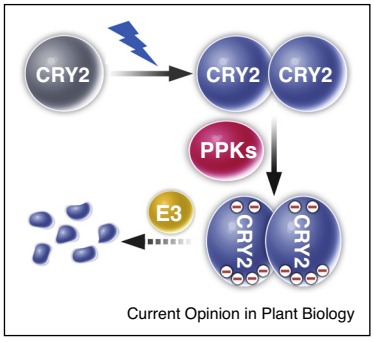
The homodimerization-dependent photoactivation and negative feedback regulation of plant cryptochromes. Cryptochromes exist as physiologically inactive monomers in darkness. Photoexcited CRY molecules undergo homodimerization to become physiologically active. The CRY homodimer or oligomers interact with the COP1/SPA complex to inhibits ubiquitylation and degradation of transcription regulators, such as HY5, Accumulation of HY5 promotes transcriptional changes of light-responsive genes, including increased transcription of *BICs* in response to light. BIC proteins interact with CRYs to inhibit CRY homodimerization, CRY activity, and photosignaling.

CRY phosphorylation

Arabidopsis CRY1 and CRY2 undergo blue light-dependent phosphorylation in plant cells [39,40]. Phosphorylation of CRY2 not only enhances its activity but also facilitates its ubiquitylation and degradation by the $CUL4^{COP1/SPAs}$ and other E3 ubiquitin ligases [39,41–43]. The label-free quantitative mass spectrometry analyses of the Arabidopsis CRY2 proteins purified from plants identified at least two dozen phosphorylated residues of CRY2, including 18 serine and 6 threonine residues [44••,45]. The level of phosphorylation in almost all those phosphorylated residues increases in response to blue light. A genetics study was performed to examine the

mutants of 13 phosphorylated residues of CRY2 [45]. As expected, the S-to-A mutations of CRY2 caused loss of phosphorylation and partial loss-of-function physiological activities. Surprisingly, the phosphomimetic S-to-D mutants of CRY2 also exhibited loss-of-function phenotype. This result may be explained by the relatively fewer negative charges carried by an aspartate (−1 charge per residue) than that introduced by phosphorylation (−1.5 to −2 charges per residue) at pH7.2 estimated for the Arabidopsis nuclear compartment. This interpretation is consistent with a hypothesis that CRY phosphorylation instigates conformational changes by a charge-dependent electrostatic repelling mechanism [46]. Although cryptochromes may undergo autophosphorylation *in vitro*, their phosphorylation *in vivo* is primarily catalyzed by protein kinases. A recent mass spectrometry analysis of the CRY2 protein complex purified from plants identified four closely related CRY2-associated protein kinases referred to as photoregulatory protein kinases (PPK1 to PPK4) [44••,47•]. PPKs are plant-specific protein kinases evolutionarily derived from the ubiquitous Casein kinase I, which were previously called MUT9-like kinases [48]. All four PPKs preferentially interact with photoexcited but unphosphorylated CRY2, and they catalyze blue light-dependent phosphorylation of CRY1 and CRY2 in human HEK293 cells co-expressing the respective CRY and PPKs [44••] (Q. Liu, unpublished results). Unexpectedly, the previously reported CRY2 kinases CK1.3 and CK1.4 did not phosphorylate CRY2 in HEK293 cells in either the electrophoretic migration shift assay or the mass spectrometry analyses [44••,49]. Therefore, the previously proposed function of plant Casein kinase I in CRY phosphorylation remains to be further investigated. In addition to CRY1 and CRY2, PPKs also have other substrates, including the phytochrome-signaling protein PIF3 [47•], histone H2A [50], histone H3 [48], and probably circadian clock proteins [51]. This makes the correlative phenotypic analyses for the specific effect of PPKs on cryptochrome phosphorylation technically difficult if not impossible. Nevertheless, the *ppk123* and *ppk124* triple mutants and the amiR^{4k} transgenic lines expressing the artificial microRNAs targeting all four PPKs exhibited delayed flowering similar to that of the *cry2* mutant [6,44••], which is consistent with PPKs being positive regulators of CRY2 function. Mass spectrometry analyses of the CRY2 proteins phosphorylated by individual PPKs indicate that different PPKs catalyze phosphorylation of CRY2 at overlapping but not identical residues, suggesting the partial functional redundancy of the four PPKs. Consistent with this hypothesis, the blue light-dependent phosphorylation of CRY1 or CRY2 appears normal in the monogenic *ppk* mutants but largely abolished in the *ppk123* and *ppk124* triple mutants and the amiR^{4k} PPK-knockdown lines. The unphosphorylated CRY2 proteins are not ubiquitylated nor degraded in the *ppk* triple mutants, confirming the previous prediction that CRY2 phosphorylation is required for its subsequent ubiquitylation and degradation [36••,39,41,45]. Taken together, those results support a model that photoexcited cryptochromes are phosphorylated by four structurally related and functionally redundant PPK kinases; phosphorylation of CRYs causes charge-dependent conformational changes to enhance the physiological activity of both CRY1 and CRY2, as well as polyubiquitylation and degradation of CRY2 (Figure 2).

Figure 2



PPKs catalyze blue light-dependent phosphorylation of CRY2 to trigger its polyubiquitylation by E3 ubiquitin ligases (E3) and degradation by the 26S proteasome.

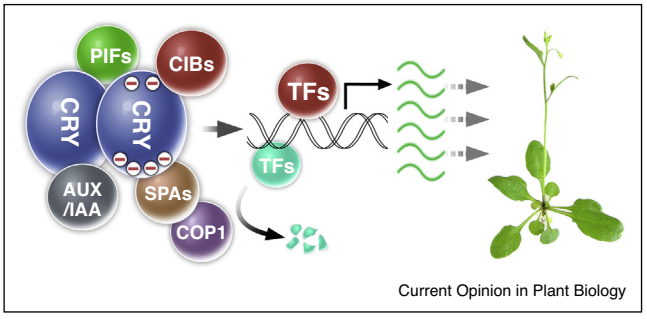
CRY signal transduction

The blue light-dependent protein–protein interactions are the primary mechanisms underlying CRY signal transduction, whereby photoexcited cryptochromes interact with regulators of gene expression to change gene expression and developmental programs (Figure 3). Similar to animal cryptochromes [52], plant cryptochromes physically interact with transcription factors to directly regulate transcription [53]. In addition, plant cryptochromes also interact with the CUL4$^{COP1\text{-}SPAs}$ E3 ubiquitin ligase and the transcription repressors AUX/IAAs to indirectly regulate transcription [53,54••].

The CRY-CIBs and CRY-PIFs complexes directly regulate transcription

The first blue light-specific CRY-interacting protein identified in plants is a bHLH transcription factor, referred to as CIB1 (cryptochrome-interacting basic-helix-loop-helix 1) [55••]. The N-terminal domain of CIB1 interacts with the PHR domain of CRY2. Because of its relatively high specificity and affinity, the blue light-dependent CRY2-CIB1 interaction has been widely utilized in the optogenetic studies of biomedical researches [56,57]. Arabidopsis has at least three other CRY2-interacting CIB1-like bHLH proteins, CIB2, CIB4, and CIB5, which form heterodimers that bind to the E-box (CANNTG) elements of the promoter of *FT (FLOWERING LOCUS T)* to activate *FT* transcription and flowering in the functionally redundant and CRY2-dependent manner [55••,58]. CRY2 mediates blue light stimulation of the

Figure 3



A model depicting interaction of CRY with three types of CRY-signaling proteins to transduce light signals. First, photoexcited CRY homodimer/oligomer interacts with transcription factors (TFs), such as CIBs and PIFs, to regulate transcription directly. Second, photoexcited CRY homodimer/oligomer interacts with transcription regulators, such as AUX/IAA, to regulate transcription indirectly. Third, photoexcited CRY homodimer/oligomer interacts with the COP1/SPA complex to suppress ubiquitylation and degradation of transcription factors and to regulate transcription indirectly. It is hypothesized that both unphosphorylated and phosphorylated CRY (depicted by negative changes) are active but the latter may have higher activity.

CIB1 activation of *FT* transcription without obvious effect on the CIB1–DNA or CIB1–chromatin interaction, suggesting an unknown mechanism underlying the CRY2 activation of CIB1. The CIB proteins are degraded by the 26S proteasome in darkness or red light, whereas blue light suppresses CIB degradation [59]. COP1, which is responsible for the proteolysis of many light-signaling proteins in darkness [60], does not seem to be involved in the regulation of CIB degradation. Surprisingly, the LOV-domain photoreceptors ZTL (ZEITLUPE) and LKP2 (LOV KELCH PROTEIN 2), but not CRY2, mediate blue light stabilization of the CIB proteins [59]. The blue light-dependent CRY2-CIBs interaction appears evolutionarily conserved, although the physiological function of CRY-CIB interaction may diverse in different plant species. For example, photoexcited CRY2 interacts with CIB1 to promote CIB1 activation of flowering in Arabidopsis, whereas CRY2 interacts with CIB1 to suppress CIB1 promotion of leaf senescence in soybean [10].

Two recent studies demonstrate that photoexcited cryptochromes also interact with the phytochrome-interacting factors PIF4 and PIF5, which are bHLH proteins in the phylogenetic clade different from that of CIBs [13•,16•,61]. It was found that photoexcited cryptochromes interact with PIF4 and PIF5, via the N-terminal PHR domain of CRYs and the N-terminal domain of PIFs in the region distinct from the phytochrome-binding motif. The CRY-PIF interaction inhibits the activity of PIF4 and PIF5, resulting in promotion of hypocotyl elongation under low blue light conditions [13•]. In addition to phytochromes, other photoreceptors have been proposed to mediate shade avoidance responses [62]. This study demonstrated the involvement of cryptochromes in this important photoresponse. It was proposed that, under low blue light, the decreased activity of cryptochromes weakens their interaction with PIFs, allowing the PIF proteins to promote stem elongation that presumably helps plants to grow out of the unfavorable shade condition under canopy. Stem elongation is favored in not only dark or shade conditions, but also relatively high ambient temperatures. The CRY-PIF interaction was found to also regulate differential growth in response to temperature changes. It was shown that CRY1 mediates blue light suppression of the high temperature-dependent hypocotyl elongation in Arabidopsis. The blue light-dependent CRY1-PIF4 interaction inhibits the transcriptional activation activity of PIF4, resulting in suppression of hypocotyl elongation of seedlings grown under blue light at high ambient temperature [16•].

The CRY-SPA/COP1 and CRY-AUX/IAA complexes indirectly regulate transcription

The COP1-SPA complex is a central regulator of plant photomorphogenesis that facilitates ubiquitylation and degradation of many light-signaling transcription factors in the absence of light. The COP1-SPA complex appears to act as the substrate adaptor of the $CUL4^{COP1\text{-}SPAs}$ E3 ubiquitin ligase, although it might also possess an intrinsic E3 ligase activity [60,63,64]. Cryptochromes interact with the COP1/SPAs complex to suppress its activity [65–69] (Figure 1). When tested in heterologous systems, CRYs interact with SPAs or COP1 in the blue light-dependent or independent manner, respectively [42,67–69]. However, light-dependent formation of the CRY1-COP1 complex was detected in plant cells [70], which may be explained by the light-dependent CRY-SPA interaction *in vivo*. The CRY-COP1/SPA interaction and CRY suppression of the COP1/SPA activity can at least partially explain the blue light-dependent stabilization of the transcription factors, such as HY5 (LONG HYPOCOTYL 5) and CO (CONSTANS) [42,67–69]. The structurally similar CRY1 and CRY2 interact with SPA1 in different ways. The C-terminal CCE domain of CRY1 interacts with the C-terminal CC-WD domain of SPA1, whereas the N-terminal PHR domain of CRY2 interacts with the N-terminal kinase-like domain of SPA1. The CRY1-SPA1 interaction suppresses SPA1-COP1 interaction [67,68], suggesting that CRY1 may act as a competitive inhibitor of COP1 [71]. In contrast, the blue light-dependent CRY2-SPA1 interaction appears to enhance the CRY2-COP1 interaction [69]. Exactly how an enhanced CRY2-COP1 interaction inhibits the activity of $CUL4^{COP1\text{-}SPAs}$ remains to be further investigated. The complexity of the CRY-COP1/SPAs interaction is further illustrated by observations that the PHR domain of CRY1 alone could trigger blue light responses [72] and that overexpression of the GUS-NC80 fusion protein,

which contains approximately 80 residues of CRY2 spanning the PHR and CCE domains, caused constitutive photomorphogenic phenotype [46]. Further studies are needed to explain exactly how CRY-COP1/SPA complexes regulate protein stability and gene expression in response to light.

AUX/IAAs are a family of transcription repressors that interact with auxin-responsive transcription factors ARFs to suppress ARF activity and transcription of auxin-responsive genes [73,74]. In response to auxin, AUX/IAAs are ubiquitylated by the $SCF^{TIR1/ABFs}$ E3 ubiquitin ligase and degraded, resulting in activation of ARFs, transcription of auxin-responsive genes, and cell elongation. It has been recently shown that photoexcited CRY1 and CRY2 interact with Aux/IAA proteins to inhibit Aux/IAA degradation and auxin signaling [54••]. It was found that blue light suppresses auxin-induced degradation of the Aux/IAA proteins and auxin-induced activation of the auxin-responsive DR5 promoter in the wild-type plants, but both responses are impaired in the *cry1* mutant plants. When the GST-tagged IAA7, IAA12, or IAA17 proteins expressed and purified from *E. coli* were incubated with extracts of seedlings treated with different wavelengths of light, these IAA proteins exhibited the blue light-specific and fluence rate-dependent interaction with photoexcited CRY1 or CRY2 [54••]. Co-immunoprecipitation assays of epitope-tagged CRY1, TIR1, and IAA17 co-expressed in protoplasts demonstrate that the CRY1-IAA17 interaction inhibits the TIR1-IAA17 interaction, suggesting that CRY1 may act as a blue light-dependent competitive inhibitor of the auxin signal transduction and hypocotyl elongation [54••].

Future perspective

Plant cryptochromes are presently known to mediate at least a dozen or more photoresponses, it remains unclear exactly how cryptochromes specifically regulate these complex photoresponses. Cryptochromes may regulate diverse aspects of plant development by interacting with close to two dozens of blue light-dependent CRY-interacting proteins, such as CIB1, CIB2, CIB4, CIB5, PIF4, PIF5, SPA1-4, BIC1-2, IAA7, IAA12, IAA17, PPK1-4. It is worth noting that the evolutionarily conserved N-terminal PHR domain of plant cryptochromes, but not the highly variable and unstructured C-terminal CCE domain that was previously thought to act as the 'signaling domain', serves as the docking domain of CRYs for almost all those blue light-dependent CRY-interacting proteins presently known. This observation implies that the PHR domain of cryptochromes, despite its highly conserved structure, may have evolved diverse structural elements to accommodate the diverse repertoire of CRY-interacting proteins. Elucidation of those structural elements would help us understand the mechanistic complexity of plant cryptochromes. Another interesting aspect of the complexity of signal transduction of plants cryptochromes concerns the interactive relationships between the photosignal and other internal or environmental signals. In this regard, the observation that PPKs interact and phosphorylate not only cryptochromes but also other proteins may provide a clue for how cryptochromes may interact with other signaling processes. It is conceivable that multiple PPK-interacting proteins may affect phosphorylation of cryptochromes in response to developmental or environmental signals other than blue light. Conversely, cryptochromes may also interact with PPKs to affect phosphorylation of other PPK-interacting proteins in response to blue light. For example, it would be particularly interesting to examine whether PPKs may phosphorylate key components of the circadian clock and whether cryptochromes may mediate blue light regulation of the phosphorylation of PPK-interacting clock proteins to alter period lengths of the circadian clock in response to light. Additional studies are apparently needed to further elucidate how cryptochromes work in plants.

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